Comparison of *in vivo* post-exercise PCr recovery and basal ATP synthesis flux for the assessment of skeletal muscle mitochondrial function

N. M. van den Broek[1], J. Ciapaite[1], K. Nicolay[1], and J. J. Prompers[1]
[1]Biomedical NMR, Department of Biomedical Engineering, Eindhoven University of Technology, Eindhoven, Netherlands

Introduction. It has been suggested that an impairment in skeletal muscle mitochondrial function plays a causative role in the development of insulin resistance and type 2 diabetes [1]. This hypothesis is predominantly based on *in vitro* measurements of for example gene expression and *in vivo* measurements of basal ATP synthesis flux with ^{31}P MR saturation transfer (ST) experiments. However, the interpretation of ^{31}P saturation transfer data is not straightforward. The lower basal ATP synthesis rates in insulin-resistant states [1, 2] could actually reflect a normal regulatory response to a lower energy demand, caused by impaired insulin signalling, rather than an impairment of intrinsic mitochondrial function [3-5]. Moreover, the Pi→ATP fluxes obtained from ^{31}P saturation transfer measurements are comprised of both ATP synthase flux and glycolytic exchange flux, with the latter contributing by as much as 80% [6, 7]. Therefore, decreased basal ATP synthesis rates do not necessarily reflect a mitochondrial defect.
From dynamic ^{31}P MRS measurements after exercise one can determine the phosphocreatine (PCr) recovery rate constant, which reflects *in vivo* muscle mitochondrial oxidative capacity because during recovery from exercise PCr is resynthesized purely as a consequence of oxidative ATP synthesis. Therefore, the post-exercise PCr recovery rate constant might be a better measure for *in vivo* mitochondrial function as compared to the basal ATP synthesis flux. In this study, both post-exercise PCr recovery and basal ATP synthesis flux were measured in a rat model of mitochondrial dysfunction and compared with oxygen consumption in isolated mitochondria.

Materials and Methods. 14-week old Wistar rats (364 ± 18 g) were given daily subcutaneous injections with either diphenyleneiodonium (DPI, an irreversible inhibitor of complex 1 of the mitochondrial electron transport chain [8]) (1.05 mg/kg body weight) dissolved in a 5% glucose solution (n = 8) or a control solution of 5% glucose (n = 8), for 10-14 days. At day 15, *in vivo* ^{31}P MRS was performed on the tibialis anterior muscle (TA) using a 6.3 T horizontal Bruker MR scanner and an ellipsoid ^{31}P surface coil (10/18 mm). ^{31}P MR spectra were acquired applying an adiabatic excitation pulse with a flip angle of 90 degrees. A fully relaxed spectrum (TR = 25 s, 32 averages) was measured at rest, followed by the ST experiment in resting TA muscle. These measurements were performed as previously reported by Jucker et al. [9]. Briefly, two spectra (TR ~ 10 s, nt = 128) were acquired for each saturation transfer experiment, one with frequency selective saturation of the γ-ATP peak (M'), and one control spectrum with saturation at a downfield frequency, equidistant from Pi (M_0) (both with a saturation pulse length of 10 s) (Figure 1A). The apparent longitudinal relaxation time of Pi (T_1') was determined by performing an 8-point inversion recovery (IR) (TI = 0.01, 1, 2, 4, 6.5, 9.5, 13 and 17 s) with γATP saturation prior to and during the inversion delay (nt = 32). The rate constant of the ATPase reaction was calculated from T_1' and the fractional reduction of Pi magnetization upon saturation of γATP according to: $k = (1\text{-}M'/M0)/T_1'$. The basal ATP synthesis flux (V_{ATP}) was then calculated by multiplying k with [Pi] at rest. After the ST experiments, time series of ^{31}P spectra (TR = 5 s, 4 averages) before, during and after muscle contractions were acquired. Muscle contractions were induced by electrical stimulation of the TA, via subcutaneously implanted electrodes. The stimulation protocol consisted of a series of stimulation pulses, applied every second, for a duration of 2 min. Recovery was followed for 15 minutes. The recovery of PCr was fitted to a mono-exponential function (Figure 1B) yielding the PCr recovery rate constant, k_{PCr}.
One day after *in vivo* MRS, animals were sacrificed and TA muscles were excised for *in vitro* measurements. One fresh TA was used for measuring oxygen consumption in isolated mitochondria with pyruvate plus malate as a substrate. Maximal (state 3) and intermediate (state I) ADP-stimulated respiration was achieved by varying ADP availability through changing Cr/PCr ratios in the assay medium. State 3 and state I respiration were compared to PCr recovery and ST experiments, respectively. The other TA was frozen in liquid nitrogen and stored at -80°C for determination of mitochondrial DNA content. Data were analyzed statistically by applying two-sided t-tests using SPSS. Level of significance was set at $p < 0.05$.
Data are presented as means ± SD.

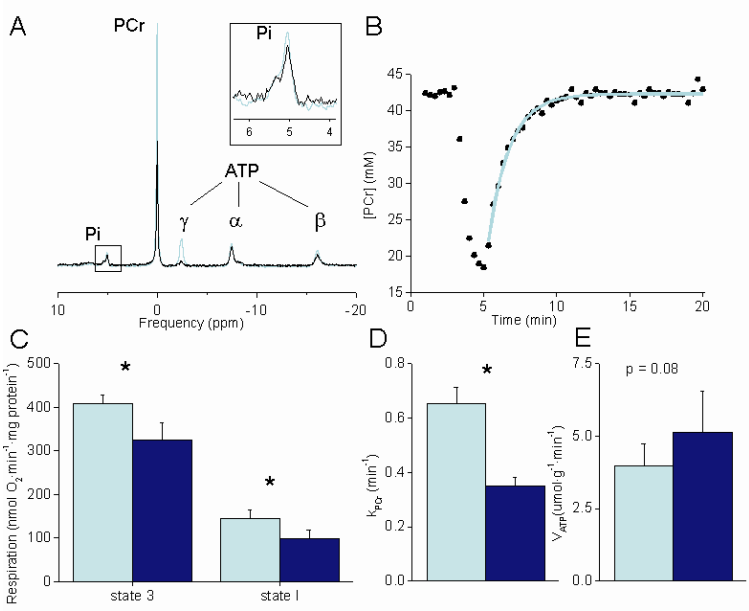

Figure 1. A) Saturation transfer spectra with saturation of γATP (black) and control frequency (blue) measured in a DPI-treated rat. B) Mono-exponential fit of PCr recovery data from a DPI-treated rat. C) State 3 and state I respiration of isolated TA mitochondria, D) k_{PCr} and E) V_{ATP} of control (light bars) and DPI-treated animals (dark bars).

Results. DPI-treated rats had a lower body weight as compared to control animals after the treatment period (375 ± 24 and 330 ± 21 g for control and DPI-treated rats, respectively). Respiration measurements showed that isolated mitochondria from DPI-treated rats consumed less oxygen when oxidizing pyruvate plus malate compared to mitochondria from control animals in both a basal (state I,144.05 ± 20.5 and 99.13 ± 18.7 nmol O_2×min^{-1}·mg protein^{-1} for control and DPI-treated rats, respectively) and a maximal respiratory state (state 3, 407.59 ± 21.39 and 325.96 ± 38.44 nmol O_2×min^{-1}·mg protein^{-1} for control and DPI-treated rats, respectively) (Figure 1C). No differences were observed in resting PCr, Pi and ADP levels and pH as determined with ^{31}P MRS. MtDNA content was similar for both groups. PCr recovery was slower in DPI treated rats compared to controls (k_{PCr} = 0.65 ± 0.06 and 0.35 ± 0.03 min^{-1} for control and DPI-treated rats, respectively) (Figure 1D). In contrast to PCr recovery, results from ST experiments did not show significant differences between groups (p=0.08) (Table 1, Figure 1E), i.e. V_{ATP} was not decreased in DPI-treated rats.

Table 1: Results ST measurements

	Control	DPI-treated
M'/M0	0.73 ± 0.06	0.67 ± 0.08
T_1' (s)	3.87 ± 0.37	3.76 ± 0.79
***k* (s^{-1})**	0.07 ± 0.02	0.09 ± 0.04
V_{ATP} (µmol/g/min)	3.96 ± 0.78	5.11 ± 1.44

Discussion and Conclusion. Two weeks of treatment with the complex 1 inhibitor DPI induced mitochondrial dysfunction, as evidenced by a decreased oxygen consumption rate in isolated mitochondria from DPI-treated rats oxidizing pyruvate plus malate. This was paralleled by a decreased *in vivo* oxidative capacity, determined from post-exercise PCr recovery. Interestingly, no significant difference in basal ST-based ATP synthesis flux was observed between DPI-treated rats and controls. This shows that saturation transfer measurements in the resting state do not necessarily reflect intrinsic mitochondrial function, but more likely reflect the ATP demand of the cell.

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